Journal of Forestry Research (2014) 25(3): 701−706
DOI 10.1007/s11676-014-0510-x

ORIGINAL PAPER

Purification of four strains of endophytic fungi from *Astragalus* and their optimized liquid fermentations

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Received: 2013-10-11; Accepted: 2013-12-23

Abstract: This study was designed to isolate endophytic fungi from *A. mongholicus* (growing in northeast China) to determine whether they can produce bioactive metabolites. Four strains of endophytic fungi (strains 16, 17, 23 and 75) were successfully isolated from *A. mongholicus* using the surface disinfection method. According to ITS-rDNA sequences analysis, strains 16 and 75 were identified as *Fusarium oxysporum*, and strains 17 and 23 were identified as *Bionectria ochroleuca*. We applied the Box-Behnken design (BBD) to optimize the liquid fermentation conditions and obtain the maximum cell dry weight (CDW) yield. Optimal parameters were obtained under the following experimental conditions: temperature of 28°C, potato dextrose agar (PDA) liquid medium of 80 mL and rotation speed of 150 rpm. The four isolated endophytic fungi did not produce astragalosides I–IV, flavonoids or polysaccharides. Isolation of additional species of endophytic fungi from *A. mongholicus* and determination of their capacity to produce biologically active substances are subjects in need of further research.

Key words: *Astragalus*, Endophytic fungi, isolation, purification, liquid fermentation

Project funding: This work was supported by grants from the Key Program of Natural Science Foundation of State (Grant No. 81274010), Heilongjiang province outstanding youth fund (Grant No. JC201101) and Talent fund of Heilongjiang University of Chinese Medicine Talent Fund.

The online version is available at http://www.springerlink.com

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Corresponding editor: Hu Yanbo

Introduction

Astragalus, the dried root of *Astragalus membranaceus* (Fisch.) Bunge or *Astragalus mongholicus* Bunge (Fabaceae) has been a famous health-promoting herb in China for more than 2000 years. Triterpenoid saponins, flavonoids and polysaccharides are believed to be the principle active constituents of *Astragalus* (Chu et al. 1988; Cho and Leung 2007), and they yield a variety of biological benefits, such as improving immunity and heart/liver functions, enhancing hypoxia tolerance and stress tolerance, promoting metabolism, reducing hypertension, regulating blood glucose and inhibiting fungal and viral infection (Ji 2012). *Astragalus* is becoming less abundant in the wild as a result of its large-scale harvest for medical uses.

Endophytic fungi are microorganisms that live in the healthy tissues of plants with no obvious effect on the host. These fungi and their host plants have co-evolved, leading the microorganisms to acquire the ability to produce bioactive substances similar to those produced by their hosts (Tan and Zou 2001; Strobel et al. 2004; Harper et al. 2007). Some of these secreted substances can be used as medicinal components for the treatment of human disease (Aly et al. 2011; de Barros et al. 2011). In recent years, 171 genera of endophytic fungi have been identified from 47 families, 81 genera and 114 species of plants (Strobel et al. 2004). Strains of endophytic fungi have been successfully isolated from *A. membranaceus* (Sun and Wang 2006; Zhou et al. 2012a; Zhou et al. 2012b) and *A. mongholicus* (Ma et al. 2012) that provide an alternative way to produce the same active compounds as their host plants. If large-scale fermentation of endo-

phytic fungi isolated from *A. membranaceus* or *A. mongholicus* can produce identical secondary metabolites, this will be an easier and more economically feasible source than wild plants, and will enable enhanced conservation of host plant species in the wild.

In this work, four strains of endophytic fungi were successfully isolated and purified from *A. mongholicus* using the surface disinfection method. The molecular analysis using the ITS-rDNA sequences was applied to construct the phylogenetic tree of these fungi. The main liquid fermentation factors including temperature, volume of potato dextrose agar (PDA) liquid medium, and rotation speed were optimized by Box-Behnken design (BBD) for obtaining the maximum cell dry weight (CDW) yield of these endophytic fungi. Subsequently, the main active ingredients of *A. mongholicus* including astragalosides I–IV, flavonoids and polysaccharides were identified in the culture liquid and mycelium of these endophytic fungi.

Materials and methods

Isolation and culture of endophytic fungi

Healthy tissues of *A. mongholicus* were collected from the experimental field of Heilongjiang University of Chinese Medicine, Harbin, China. Fresh clean *A. mongholicus* taproots were cut into segments of about 3 cm. Segments were rinsed with 75% alcohol for 3 min, washed twice for 2 min in sterile water, immersed in 10% NaClO for 7 min, and again washed twice for 2 min in sterile water. All segments other than the tips of the original taproot were then cut into pieces under aseptic conditions. Four or five pieces of taproot were placed on PDA medium. *A. mongholicus* taproots were incubated in a homoeothermic incubator at 30°C for 3–7 days. The hyphal tip was removed and placed on new PDA, incubated at 30°C and repeated until a pure culture was obtained. Unbroken taproots, 0.1 mL sterile water from the final wash, and imprints from the cut pieces were cultured as controls under the same conditions used for the experimental samples.

Strain identification

The selected four strains of endophytic fungi were evaluated by the phylogenetic analysis of their ITS-rDNA sequences. Total DNA was extracted and purified using a fungil DNA Mini Kit (Meilian Biotech, Shanghai, China) according to the manufacturer's instructions. The fungal ITS-rDNA fragments was amplified by polymerase chain reaction (PCR) using a pair of universal primers including 5′-TCCACCAGCTKYGAGAACTC-3′ and 5′-ACCTCCTTCATGGAGACCTT-3′. PCR products were purified using a UNIQ-10 Spin Column PCR Products Purification Kit (Gentaur, Kampenhout, Belgium). Sequencing of the PCR products was performed by the service of Sangon Engineering Technology and Service Co. Ltd. (Shanghai, China). The ITS sequence of each isolate was compared with the data available in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to estimate the phylogenetic relationships of the endophytic fungi. The neighbor joining (NJ) phylogenetic tree was constructed from evolutionary distance data by MEGA 4.0 software. The bootstrap was 1000 replications to assess the reliable level to the nodes of the tree. The NJ tree was estimated using pairwise genetic distances based on all substitutions with the Jukes−Cantor distance parameter.

Optimization of fermentation conditions

We selected four strains of *A. mongholicus* endophytic fungi for the liquid fermentation culture based on the characterization of good growth performance. These four endophytic fungi were grown on PDA at 30 °C for 5 days and then inoculated into 250-ml Erlen-meyer flasks containing PDA liquid medium. The fermentation lasted for 8 days under a range of conditions according to the experimental design. To achieve the optimum fermentation of four strains of fungi, BBD was applied to survey the effects of three key independent variables at three levels (temperature 25–31 °C, volume of PDA liquid medium 60–100 mL, and rotation speed 120–180 rpm) on the dependent variable (CDW yield of each endophytic fungi). A total of 17 randomized experiments including 14 factorial and 3 zero-point tests were designed. Regression analysis was carried out to evaluate the response function as a quadratic polynomial:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j \ (k = 3) \quad (1)$$

where, Y is the predicted response; β_0, β_j, β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively; X_i and X_j are the independent coded variables; and k represents the number of variables. The actual and coded levels of the independent variables used in the experimental design are summarized in Table 1. The experiment data were analyzed statistically with Design-Expert 7.0 (State-Ease, Inc., Minneapolis MN, USA). Analysis of variance (ANOVA) was performed to calculate and simulate the optimal values of the tested parameters.

Determination of active metabolites produced by endophytic fungi

The fermentation process was performed under the conditions of 80 mL PDA liquid medium, 150 rpm, 28°C and 8 days, and the cultures were then separated into culture liquid and mycelium by filtration. The culture liquid was extracted three times with an equal volume of n-butanol. The oven-dried mycelia were resuspended in deionized water, then subjected to ultrasonic processing for 30 min and extracted three times with an equal volume of n-butanol. The dry extracts were obtained by evaporation of the organic solvent and dissolved in methanol for further analysis. *A. mongholicus* astragalosides I–IV were determined using the LC–MS/MS method reported by Zu et al. (2009). *A. mongholicus* flavonoids were determined using the Salkowski reaction method. *A. mongholicus* polysaccharides were determined using the phenol-sulfate acid method.

Statistical analysis

Results were expressed as means ± standard deviations. The data were statistically analyzed using SPSS statistical software, version 17.0 (SPSS Inc, Chicago, Illinois, USA). Differences between means were determined by analysis of variance (ANOVA) with Duncan's test on the level of significance declared at $p < 0.05$.

Results

There was no microorganism growth in the control samples, demonstrating that the fungi in the experimental samples had been isolated from *A. mongholicus* tissue. In this work, a total of twenty-eight strains of endophytic fungi were isolated, but only four strains (strains 16, 17, 23, and 75) showed stable growth, exuberant vitality and good repeatability. Each strain exhibited variation in colony morphology, color and microstructure (Fig. 1). Subsequently, the isolated endophytic fungi were identified by sequencing the internal transcribed spacers (ITS) of the rDNA region. The length of the amplified rDNA fragment ranged from 500 to 600 bp. After the BLAST searches, ITS-rDNA sequences of strains 16 and 75 were most closely related to *Fusarium oxysporum*, and ITS-rDNA sequences of strains 17 and 23 were most closely related to *Bionectria ochroleuca* (Fig. 2).

To obtain the optimum fermentation conditions for the four isolated strains of endophytic fungi, the temperature, volume of PDA liquid medium, and rotation speed were optimized by BBD. The experimental design matrix and all the relevant data are illustrated in Table 1. The ANOVA results of the built quadratic model are presented in Table 2. Significance levels of all models ($P < 0.05$ in all cases) and desirable determination coefficients ($R^2 \geq 0.9190$ for all models) suggested that all the built mathematical models were precise and applicable. The second-order polynomial models were described by the following equations (ignoring insignificant items):

$$Y_{16} = 0.27 + 0.017X_2 + 0.014X_1X_2 - 0.011X_2X_3 - 0.16X_1^2 - 0.017X_2^2 - 0.016X_3^2 \quad (2)$$

$$Y_{17} = 0.39 - 0.037X_1 + 0.029X_2 - 0.082X_1^2 - 0.061X_2^2 - 0.054X_3^2 \quad (3)$$

$$Y_{23} = 0.26 - 0.011X_1 + 0.018X_2 + 0.012X_3 - 0.013X_1X_3 + 0.019X_2X_3 - 0.075X_1^2 - 0.066X_2^2 - 0.051X_3^2 \quad (4)$$

$$Y_{75} = 0.37 + 0.015X_1X_3 - 0.015X_2X_3 - 0.022X_1^2 - 0.048X_2^2 - 0.039X_3^2 \quad (5)$$

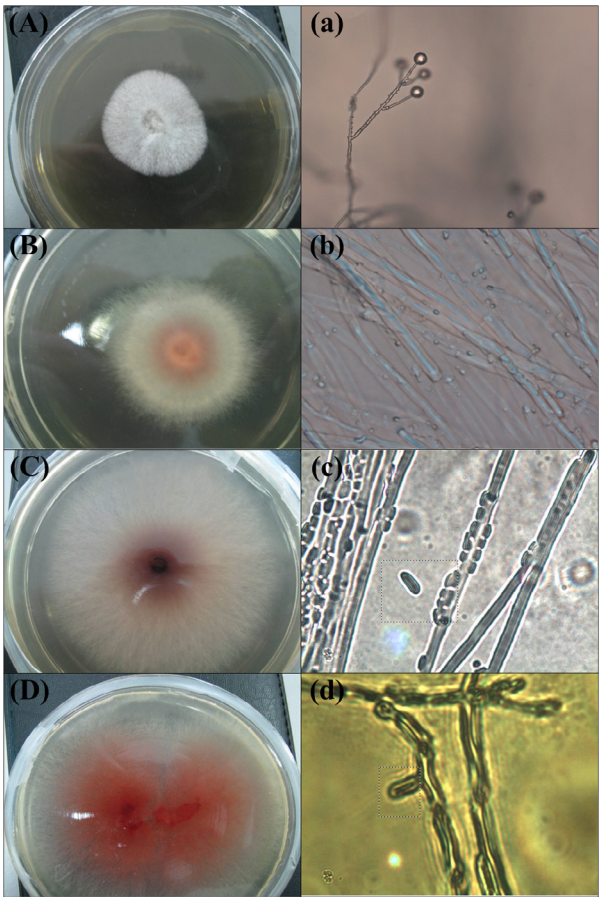

Fig. 1: Colony morphology and microscopic characteristics of 16 (A, a), 17 (B, b), 23 (C, c), 75 (D, d) strains of endophytic fungi isolated from *A. mongholicus*.

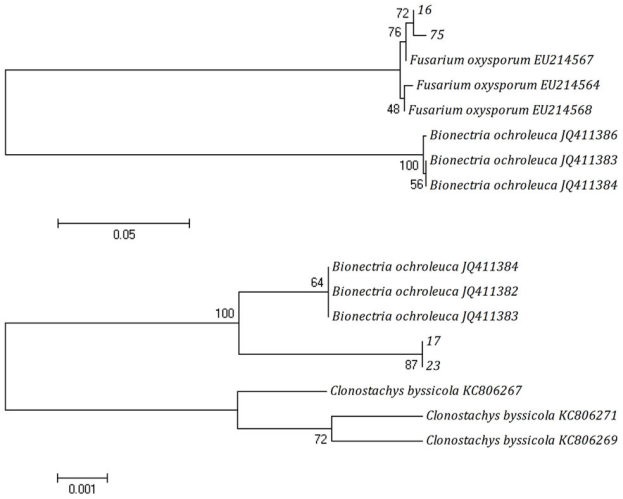

Fig. 2: Phylogenetic tree constructed by the program neighbour joining (NJ) based on ITS1-5.8S-ITS2 sequences of endophytic fungi isolated from *A. mongholicus*. Bootstrap values (1000 tree interactions) are indicated at the nodes.

Table 1: Results of CCD for the production of CDW during the liquid fermentation process.

Runs	X_1 (T [a], °C)	X_2 (V [b], mL)	X_2 (R [c], rpm)	CDW for each strain (kg)			
				16	17	23	75
1	0 (28)	0 (80)	0 (150)	0.2689	0.3901	0.2587	0.3635
2	0 (28)	1 (100)	1 (180)	0.2353	0.2877	0.1989	0.2546
3	0 (28)	1 (100)	-1 (120)	0.2635	0.3064	0.1228	0.2879
4	1 (31)	-1 (60)	0 (150)	0.2022	0.1942	0.0951	0.2725
5	1 (31)	0 (80)	-1 (120)	0.2425	0.2010	0.1346	0.2744
6	-1 (25)	-1 (60)	0 (150)	0.2297	0.2275	0.1089	0.2799
7	0 (28)	-1 (60)	1 (180)	0.2310	0.2703	0.1214	0.2993
8	-1 (25)	1 (100)	0 (150)	0.2414	0.3261	0.1562	0.3004
9	0 (28)	-1 (60)	-1 (120)	0.2139	0.2347	0.1198	0.2716
10	0 (28)	0 (80)	0 (150)	0.2651	0.3891	0.2578	0.3664
11	1 (31)	1 (100)	0 (150)	0.2697	0.2404	0.1088	0.3298
12	0 (28)	0 (80)	0 (150)	0.2747	0.3869	0.2585	0.3647
13	0 (28)	0 (80)	0 (150)	0.2664	0.3947	0.2578	0.3701
14	-1 (25)	0 (80)	1 (180)	0.2291	0.3149	0.1562	0.3057
15	0 (28)	0 (80)	0 (150)	0.2691	0.3912	0.2581	0.3628
16	1 (31)	0 (80)	1 (180)	0.2354	0.2168	0.1167	0.3184
17	-1 (25)	0 (80)	-1 (120)	0.2429	0.2836	0.1205	0.3222

[a] T is expressed as the temperature (°C), [b] V the volume of liquid medium (mL), [c] R the rotation speed (rpm).

Table 2: ANOVA results of the quadratic model for the production of CDW.

Modle	Strains			
	16	17	23	75
R^2	0.9801	0.9880	0.9902	0.9190
R^2 adjusted	0.9546	0.9725	0.9775	0.8150
P-value [a]	0.0001	0.0001	0.0001	0.0045
Adequate precision	17.722	21.369	21.675	8.612

[a] *P*-value less than 0.05 indicate model term is significant.

[b] Variation of the data around the fitted model.

Where Y_{16}, Y_{17}, Y_{23} and Y_{75} are the CDW yields of strains 16, 17, 23, and 75 of endophytic fungi, respectively; X_1 is the temperature (°C); X_2 is the volume of PDA liquid medium (mL); and X_3 is the rotation speed (rpm). Based on the above mathematical models and considering the experimental methods, the optimal liquid fermentation parameters were as follows: temperature of 28°C, PDA liquid medium of 80 mL and rotation speed of 150 rpm. Under the optimized fermentation conditions, maximum CDW yields for strains 16, 17, 23, and 75 were 0.2699 kg, 0.3945 kg, 0.2587 kg, and 0.3634 kg, respectively.

As the LC-MS/MS results show in Fig. 3, astragalosides I–IV were not identified in the culture liquids or mycelium extracts of the four isolated endophytic fungi. For the detection of flavonoids, hydrochloric acid and magnesium powder reaction indicated that pink was found in the *A. mongholicus* sample and rutin standard solutions, while no color change was observed in the culture liquids or mycelium extracts of the four isolated endophytic fungi (data not shown). The results of phenol-sulfuric acid tests showed that red precipitate was found in the *A. mongholicus* sample solution but not in the culture liquids or mycelium extracts of the four isolated endophytic fungi (data not shown). Our results show that neither astragalosides I–IV, flavonoids nor polysaccharides were identified in the culture liquids or mycelium extracts of the four isolated endophytic fungi.

Discussion

Three imortant and innovative outcomes resulted from this study: (1) four strains of endophytic fungi were screened and isolated from *A. mongholicus*, each showing stable growth, exuberant vitality and good repeatability in the culture medium; (2) molecular phylogenetic analysis of strains 16 and 75 were most closely related to *F. oxysporum*, and strains 17 and 23 were most closely related to *B. ochroleuca*; (3) the maximum yields of CDW for strains 16, 17, 23, and 75 were 0.2699 kg, 0.3945 kg, 0.2587 kg and 0.3634 kg, respectively, under the optimal liquid fermentation conditions of temperature (28°C), PDA liquid medium (80 mL) and rotation speed (150 rpm).

The isolated strains 16 and 75 were genetic identical to *F. oxysporum*, and strains 17 and 23 were genetic identical to *B. ochroleuca*. No previous reports documented isolation of these two species of endophytic fungi strains from *A. mongholicus* (growing in northeast China). It is reported that *F. oxysporum* is a common species of endophytic fungi existing in various plants, such as *Cinnamomum kanehirae* (Wang et al. 2011), *Ginkgo biloba* (Cui et al. 2013), *Catharanthus roseus* (Kumar et al. 2013) and *Lilium lancifolium* (Liu et al. 2012). Recently, *B. ochroleuca* has been successfully isolated from *Nothapodytes foetida* for producing antimicrobial and free radical scavenging metabolites (Samaga et al. 2013)

In the previous reports, many strains of endophytic fungi isolated from ginseng root and flower produced active compounds, such as ginsenoside Rb1, ginsenoside Rd, ginsenoside Re, xanthatin, isotanshinone II, ginseng falcarinol, 2,4,5- trimethyl-1,3-dihydroxyhenzene, 2,4-dihydroxyl-3,5,6- methyl 3-(trifluoromethyl) benzoate, mannitol, green mycophenolic acid, ergosterol, peroxy ergosterol, hydroxyethyl phenol and brefeldin A (Chen 2007; Sun et al. 2008; Park et al. 2012). Additionally, a number of endophytic fungi isolated from yew stems and roots produces paclitaxel (Liu et al. 2009). In the co-culture of ginsenoside Rb_1 with three strains of panax endophytic fungi for seven days, the endophytic fungi transformed ginsenoside Rb_1 into ginsenoside Rd (Chen 2007). In the co-evolution process with plants, endophytic fungi not only produce special chemicals, but also induce the formation and growth of some metabolites in host plants, particularly in medicinal plants. After the endophytic fungi isolated from *Dracaena cochinchinensis* stem were inoculated into the living *D. cochinchinensis*, the amount of *Sanguis draconis* increased by 66−120% (Ou et al. 2013).

The above reports show that many endophytic fungi can synthesize the same natural products that occur in host plants. Kusari et al. (2008) hypothesized that the production of these compounds in host plant does not result exclusively from metabolic

processes of endophytic fungi but is rather the consequence of concomitant plant and fungal biosynthesis. The gene for biosynthesis of some active compounds might be transferred between host plant cells and endophytic fungi by horizontal gene transfer. Strobel (2002) proposed that endophytic fungi might have developed genetic systems promoting the transfer of information between themselves and the host plant during the long co-evolution process.

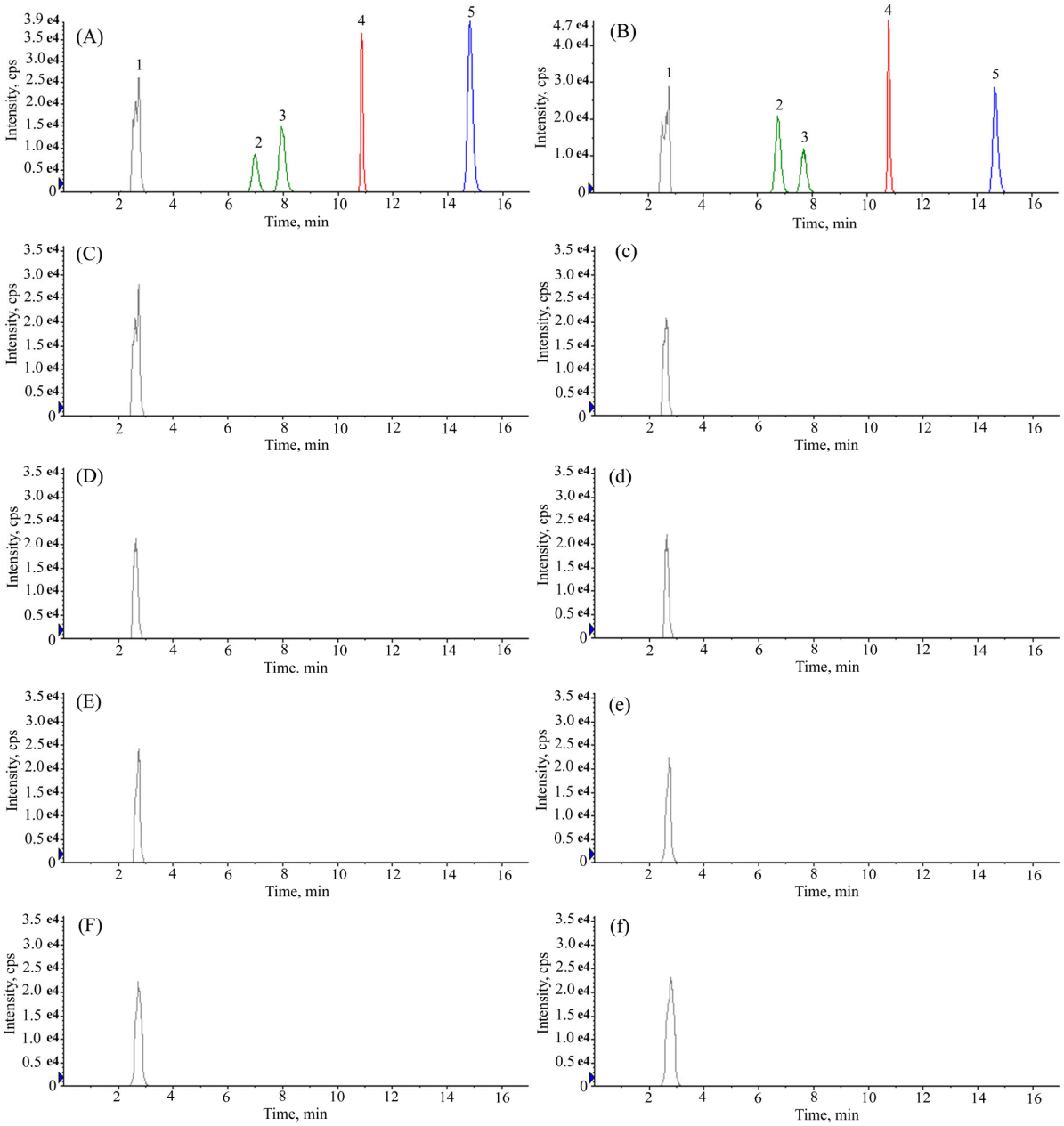

Fig. 3: LC-MS/MS chromatograms of standard mixture (A), *A. mongholicus* sample (B), culture liquid of 16 (C), 17 (D), 23 (E) and 75 (F) strains, and mycelium extracts of 16 (c), 17 (d), 23 (e) and 75 (f) strains. 1, Internal standard; 2, astragalosides IV; 3, astragalosides III; 4, astragalosides II; 5, astragalosides I.

To our best knowledge, there are few reports of endophytic fungi from *A. mongholicus* (growing in northeast China) producing active substances. It is known that the main active ingredients of *A. mongholicus* are astragalosides I–IV, flavonoids and polysaccharides. In this study, astragalosides I–IV, flavonoids and polysaccharides were not isolated in culture liquids or in mycelium extracts of the four selected endophytic fungi. This work was the first investigation of production of active substances from endophytic fungi of A. mongholicus in northeast China. Other bioactive components from the four selected endophytic fungi will be further clarified in further studies. In addition, fermentation of a mixture of species of endophytic fungi will also be conducted in future based on the presence of multiple species of fungi in the tissues of wild *A. mongholicus.*

Conclusions

In this study, we successfully isolated and purified four strains of endophytic fungi (strains 16, 17, 23, and 75) from *A. mongholicus*, and subjected them to phylogenetic analysis. The liquid fermentation parameters of the four isolated fungi were optimized by BBD. Under the optimal conditions of temperature (28°C), PDA liquid medium (80 mL), and rotation speed (150 rpm), the maximum CDW yields for strains 16, 17, 23, and 75 were 0.2699, 0.3945, 0.2587, and 0.3634 kg, respectively. Astragalosides I–IV, flavonoids and polysaccharides were not identified in culture liquids or in mycelium extracts of the four selected endophytic fungi. Moreover, isolation of additional species of endophytic fungi from *A. mongholicus* or other host plant species in northeast China and determination of their capacity to produce biologically active substances are subjects in need of further research.

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